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Thesis

THE ANTIGENIC PROPERTIES OF SYNTHETIC GELATIN COMPOUNDS

by

Morris Nathan Green

(S.B., Massachusetts Institute of Technology, 1933)

submitted in partial fulfilment of the
requirements for the degree of
Master of Arts

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1.

I. Review of the literature.

A. The non-antigenicity of gelatin.

Most proteins are antigenic. Gelatin has repeatedly been shown to be non-antigenic.

One of the first observations made to show that gelatin is non-antigenic was by Wells.²⁴ He tried anaphylaxis experiments on guinea-pigs and got negative reactions. He believed that gelatin was^{non} antigenic because it lacked aromatic amino acids and would not therefore produce toxic portions according to Vaughan's Theory of Anaphylaxis.

¹⁷
Pabes and Regazzi sought to induce anaphylactic reactions in rabbits by repeatedly injecting both normal and hydrolysed gelatin and obtained negative results.

⁸
Kahn and McNeil attempted to get complement fixation reactions by injecting gelatin. They injected rabbits intravenously at twenty-four hour intervals with successive doses of 50, 75 and 100 milligrams of gelatin and bled eight days after the last injection. They obtained negative results. This is not surprising in view of the fact that their doses were large and only a few were given.

The most systematic and exhaustive work ever done to show the non antigenicity of gelatin was reported by Starin.²¹

For the preparation of his antigens, Starin purified a sample of the French "Gold Leaf" gelatin by repeated precipitation in alcohol and tested the resulting product for protein impurities. Since this method may be of some interest I quote from Starin:

2. The non-antigenicity of gelatin.

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Kahn and Kassel attempted to get complement fixation reactions by injecting gelatin. They injected rabbits intravenously at twenty-four hour intervals with successive doses of 50, 75 and 100 milligrams of gelatin and blood eight days after the last injection. They obtained negative results. This is not surprising in view of the fact that their doses were large and only a few were given.

The most systematic and exhaustive work ever done to show the non-antigenicity of gelatin was reported by Hirst.

For the preparation of his antigens, Hirst utilized a mixture of the so-called "Gold Bead" gelatin by repeated precipitation in alcohol and tested the results by various methods. He found that this method may be of some interest.

"Gelatin can be separated and quantitatively determined by adding to the gelatin solution a mixture of one part of picric acid (sat. aq. sol.) plus four parts of absolute alcohol. At 80°C all other proteins are precipitated while the gelatin is held in solution. On evaporation of the alcohol following filtration, the gelatin is precipitated quantitatively, as cooling progresses. The application of this method to the product obtained, gave no indication of the presence of proteins other than gelatin."

Using the protein purified as above, Starin carried out anaphylaxis, complement fixation, precipitin and meiostagmin reactions. He also tried to demonstrate the production of proteolytic ferments.

For the anaphylaxis experiments, he used rabbits, guinea-pigs and dogs. The animals were observed for the following symptoms: (a) general (b) variations in temperature following a second infection (c) character of the blood pressure (in dogs) (d) changes in the coagulation time of the blood. (e) necropsy findings.

The rabbits were sensitized with intraperitoneal doses of gelatin ranging from 0.04-0.1 of a gram. The second and third injections were 5-10 times the sensitizing doses and were given both intravenously and intraabdominally allowing 10, 12, and 16 days to intervene between injections. Only doubtful and negative symptoms were observed.

Starin also makes the interesting observation that the intravenous injection of more than 0.5 gram of gelatin is

Gelatin can be prepared and quantitatively determined by adding to the gelatin solution a mixture of one part of picric acid (sat. sol.) plus four parts of absolute alcohol. At 80°C all other proteins are precipitated while the gelatin is held in solution. On evaporation of the alcohol following filtration, the gelatin is precipitated quantitatively as cooling progresses. The application of this method to the product obtained, gave no indication of the presence of proteins other than gelatin.

Using the protein purified as above, serum settled out anaphylactic, complement fixation, precipitin and melanin reactions. It also gives a demonstrable precipitation of

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For the anaphylactic reaction, the following symptoms: (a) general (b) variations in severity following a second injection (c) character of the blood serum in dogs (d) changes in the aggregation time of the blood. (e) response to the toxin. The toxin was sensitized with intraperitoneal doses of gelatin ranging from 0.25-0.1 or more. The second and third injections were 5-10 times the sensitizing dose and were given both intravenously and intraperitoneally following 10, 15, and 16 days to intervals between injections. Only control and negative symptoms were observed. It is also made an interesting observation that the intraperitoneal injection of more than 0.5 gram of gelatin is

fatal to rabbits.

The anaphylaxis experiments with guinea pigs were carried out in the same general way except that smaller doses were given (0.005-.05 grams). The routes of injection were intra-abdominally, intracardial and intrajugular. Only doubtful and negative symptoms were observed.

Two young dogs were also injected intraabdominally with initial doses of 0.1 to 0.5 gram of gelatin followed by doses of 2 and 3 grams through the femoral vein. No significant changes were observed in the blood clotting time of the dogs, nor did any necropsy findings indicate anaphylaxis.

Starin also tried to produce precipitins against gelatin using both the intraabdominal and intravenous routes singly and together. The total amount of gelatin injected varied from 0.6 to 12 grams. The period of injection varied from a few days to 9 months and the number of doses from 3^{to} 25. Precipitin and complement fixation tests were consistently negative.

Since the meiostagmin reaction and the production of proteolytic ferments have fallen into some disrepute as criteria of antigenicity they will not be discussed here.

Arthus², however, reported that he was able to sensitize rabbits with gelatin by intravenous injection. After five or six injections, he observed lack of absorption of the injected material and abscess formation, at the point of injection, such as is found in local anaphylaxis or the Arthus phenomenon.

⁴
Bruynoghe and Vassiliadis found positive complement fixation and negative precipatation using gelatin as an antigen. They,

later to rabbits.

The anaphylaxis experiments with guinea pigs were carried out in the same manner as except that smaller doses were given 0.005-0.02 grams. The results of injection were invariably negative, intracutaneous and intraperitoneal, only doubtful and negative symptoms were observed.

The young dogs were also injected intracutaneously with initial doses of 0.1 to 0.5 gram of gelatin followed by doses of 1 to 5 grams through the femoral vein. No significant changes were observed in the blood during time of the dogs, nor did any anaphylaxis develop.

Statin also tried to produce reactions against gelatin using both the intracutaneous and intravenous routes singly and together. The total amount of gelatin injected varied from 0.5 to 25 grams. The period of injection varied from 2 to 10 days to 2 months and the number of doses from 1 to 10. Reactions and toxic shock reactions were consistently negative.

Since the main reaction and the production of protein is known to have taken into the blood stream as observed at anaphylaxis, we tried to produce anaphylaxis.

First, however, reported that he was able to produce reactions with a series of intravenous injections. After 1/2 or 1/4 injections, he observed lack of sensation of the injected material and observed formation of the solid of injection.

4
Trigono and Vassiliades found positive results in anaphylaxis and negative precipitation values were in an anaphylaxis.

however, used a very large antigenic "dose", One half of the largest quantity of gelatin which when coupled to anilin will allow complete hemolysis in a hemolytic system.

Using antisera to gelatin and anilin-gelatin they did not get fixation with anilin-gelatin with either antiserum but gave fixation with gelatin alone. These results are very peculiar and due to the lack of specific details cannot be evaluated properly.

Despite a few discrepancies in the literature, the consensus of opinion is that gelatin is non-antigenic. The very careful work of Starin is especially convincing. It must be remembered, however, that the gelatin used in these experiments was not all from the same source. All gelatins are not of the same composition. They will vary with the type of collagen used in its preparation, and the method of hydrolysis, among other factors. Gelatin is not a single chemical entity as has been demonstrated by Krishnanurti and Svedberg.⁹ From an ultracentrifugal study of gelatin solutions, they find that gelatin solutions contain a mixture of proteins varying in molecular weight from 11,000 to 70,000.

Furthermore, not all investigators have taken precautions to separate foreign proteins from the gelatin. These considerations might explain some of the discrepancies in the literature.

however, used a very large amount of "base", and all of the
largest amount of gelatin which was added to make with
after complete hemolysis in a hemolytic solution.

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factor. Gelatin is not a single chemical entity as has been
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structural study of gelatin solutions, it is found that gelatin
molecules contain a mixture of proteins varying in molecular
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Furthermore, not all these proteins have been shown
to separate from the gelatin. These
conclusions might explain some of the discrepancies in the
literature.

B. Compounds related to gelatin.

In connection with the non-antigenicity of gelatin it is interesting to note that glue, a substance related to gelatin is non antigenic, as has been demonstrated by Ramsdell and Walzer. Since glue is a potent atopen in human supersensitive-¹⁸ness, they investigated its antigenic effect in the guinea-pig and rabbit. Using the Dale method they obtained only weak and submaximal contractions in the uterine strips from injected animals. They conclude therefore that glue is non antigenic.

¹⁴Loiselleur and Urbain found collagen to be antigenic. They prepare collagen according to the directions of Nageatte. This method has the advantage that no heat is required to dissolve the collagen thereby eliminating one cause of denaturation. They prepare the collagen as follows:

The tendons of a rat's tail were suspended in a very dilute solution of acetic acid and allowed to swell; the collagen dissolved in the acid was separated from the cell residue by centrifuging and preserved on ice in the presence of ether. The solution had a pH of 3.89 and its protein content was 1.93 grams per liter.

Two rabbits received 6 subcutaneous injections of collagen from 1-6 cc. After the fifth injection the edema characteristic of an Arthus phenomenon appeared. At this point the complement fixation reaction became positive. The fixation tests were controlled by using rat serum as an antigen. No fixation was obtained with this control. The authors therefore conclude that the reaction is specific for collagen.

In connection with the non-availability of gelatin is is interesting to note that since a substance related to gelatin

is non-analytic, as has been demonstrated by Klemm and others. Since this is a potent factor in human experimental work, they investigated its analytic effect in the human system and results. Using the same method they obtained only weak and

substantial reactions in the human system from injected animals. They conclude therefore that this is non-analytic. Gelatin and uric acid found collagen to be analytic.

They prepare collagen according to the direct one of Haggard. This method has the advantage that no heat is required to dissolve the collagen thereby eliminating the cause of decomposition. They prepare the collagen as follows:

The formula of a rat's tail were prepared in a test tube. The solution of acids used and added to water; the

collagen dissolved in the acid was prepared from the tail

collagen by digestion and extraction on the water-bath at 40°C. The solution had a pH of 5.5 and 10% protein content.

and 1.5% glucose per liter.

Two rabbits received a small amount of collagen from 1-5 cc. After the first injection the second was given of an allergic phenomenon appeared. At this point the experiment was terminated. The reaction became positive. The time on tests was

controlled by using rat serum as an antigen. The reaction was obtained with this control. The reaction between collagen

and the reaction as well as the collagen.

They also tested the action of radium on this antigen and found that irradiation decreased the viscosity and also reduced the antibody titre to one-third of the original; the same effects are obtained by heating the antigen at 100°C .

These results are at present **unconfirmed**. If confirmed, it would cause a fundamental revision of our ideas concerning the non-antigenicity of gelatin. This point will be discussed later.

They also tested the action of raising the pH and found that irradiation decreased the viscosity and also reduced the antibody titre to one-third of the original; the same effects are obtained by heating the antigen at 100°C. These results are at present unconfirmed. It would seem that a fundamental revision of our ideas concerning the non-specificity of gamma rays. This point will be discussed later.

C. Synthetic gelatin compounds as antigens.

Although gelatin itself is non-antigenic there are some reports in the literature in which gelatin has been made antigenic by coupling with aromatic groups.

The theoretical background for this work is discussed by Wells.^{23b} From the many analyses of gelatin that have been made, it has been found that gelatin is characterised by a lack of aromatic radicals. It contains no tryptophan or tyrosin and only a very small amount of phenylalanine. Since most antigenic proteins contain these amino-acids, it has been thought that the non-antigenicity of gelatin is due to a lack of these amino acids. In support of this contention are the following facts:

23b

(1) It has been shown by Vaughan that in the toxic and non-toxic portions formed in the cleavage of proteins, the toxic fractions contain aromatic radicals. Gelatin yields no toxic fraction because it is deficient in aromatic groups.

(2) Proteins deficient in amino acids other than aromatic radicals are antigenic. For instance, zein lacks tryptophan, lysin and glycine, but has a large proportion of tyrosin. Gliadin lacks glycine, while casein is deficient in cystin and glycine. Still, all these proteins are strongly antigenic.

(3) The protamins are non antigenic but they consist mostly of diamino acids and a small quantity of a few of the mono-amino acids. Lysin is not present in the protein, hordein as well as arginin and histidin. This indicates that the diamino acids are not of importance in determining antigenicity.

...the following results have been obtained...

Although it is not a simple matter to determine the exact amount of protein in the literature in which it is mentioned, there are some reports in the literature in which it is mentioned that it is made up of protein with aromatic groups.

The theoretical basis for this work is discussed by ... from the many analyses of protein that have been made, it has been found that protein is characterized by a large amount of aromatic radicals. It contains no nitrogen or phosphorus and only a very small amount of other radicals. It has been found that protein contains these amino acids, it has been found that the non-antigenicity of protein is due to a lack of these amino acids. In support of this conclusion are the following results:

2.3

(1) It has been shown by Yaguchi that in the case of non-antigenic portions formed in the cleavage of proteins, the amino acids which are released are aromatic radicals. Protein is made up of protein with aromatic groups. It is found on digestion that it is deficient in aromatic groups. (2) Protein is deficient in amino acids other than aromatic radicals and nitrogenous. For instance, when it is digested, it is deficient in glycine, but has a large proportion of aromatic radicals. It is deficient in glycine and alanine. In fact, all these proteins are strongly antigenic.

(3) The proteins are not antigenic but they contain mostly of amino acids and a small amount of a few of the amino acids. It is not present in the protein, however, as in the case of antigenic proteins. This indicates that the amino acids are not of importance in determining antigenicity.

(4) It has been shown by Obermeyer and Pick that the aromatic radicals of proteins are important in determining specificity. This is effected by introducing into the protein molecule substances which are known to react with the benzene rings. Proteins thus altered lose their species specificity entirely.

If aromatic radicals are necessary for antigenicity, it follows that their introduction into the protein molecule might make gelatin antigenic. This has been attempted by a few investigators.

Adant¹ claims to have produced precipitins to gelatin-diazoanilin. Using anti-gelatin-diazo-anilin serum, he obtained precipitins with both the diazo compound and gelatin, although the latter was not antigenic. He also obtained specific inhibition of precipitation with other anilin proteins.

Bruynoghe and Vassiliadis⁴ observed that anti-gelatin-diazo-anilin fixed complement in the presence of gelatin but not with the homologous antigen.

Hooker and Boyd⁵ found that anti-gelatin-diazo-arsanilic acid gave precipitin reactions with both casein-diazo-arsanilic acid and ovalbumin diazo-arsanilic acid but not with the homologous antigen. Anti-gelatin-diazo-arsanilic acid also fixed alexin in the presence of casein and egg-white-arsanilic acids. The antiserum was specifically inhibited by both phenol-diazo-arsanilic acid as well as by ^{the} homologous compound.

They reach the conclusion that "antigenicity may be conferred upon gelatin by coupling it with an unsaturated cyclic

It has been shown by Scheraga and that the aromatic radicals of proteins are important in determining antigenicity. This is affected by introducing into the protein molecule substances which are known to react with the benzene rings. Proteins thus altered lose their species specificity entirely.

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Abant claims to have produced precipitates to gelatin-antigen. Using anti-gelatin-diastase serum, he obtained precipitates with both the diastase compound and gelatin. Although the latter was not antigenic. He also obtained specific inhibition of precipitation with other animal proteins. Brunschwig and Vassilakis observed that anti-gelatin-diastase serum complement in the presence of gelatin but not with the homologous antigen.

Hooker and Boyd found that anti-gelatin-diastase serum would give precipitin reactions with both diastase-diastase-antigen and ovalbumin diastase-antigenic but not with the homologous antigen. Anti-gelatin-diastase serum also failed to react in the presence of casein and egg-white-antigenic antigen. The antigen was specifically inhibited by both diastase-antigen and ovalbumin diastase-antigenic serum.

compound" but add that the results, "do not show that non antigenicity of gelatin is due solely to its deficiency in aromatic amino-acids." Other investigators using different prothethic aromatic groups were not able to confer antigenicity on gelatin but obtained only combining effects similar to haptens.

¹⁰ Landsteiner found that diazotised metanilic acid coupled with gelatin gave precipitins with anti-horse-serum coupled with m-diazo-henzenesuphonic acid. This gelatin compound specifically inhibited reactions between the diazo proteins and its antiserum using gelatin diazo-arsanilic acid. He obtained the same results with the homologous antihorse serum compound.

¹⁵ Medveczky and Uhrovits found that henzoylated gelatin did not give reactions with its antiserum, but give very strong reactions with antisera for henzoylated horse serum and benzlylated typhoid bacilli. The antiserum for benzoylated bacilli reacted with a solution of gelatin containing 5 per cent of the henzoyl radical in a titer of 1: 10,000,000.

Benzoylated gelatin was strongly anaphylactic to guinea pigs sensitized with the benzoylated typhoid bacilli, so that a dose of 0.0000 15 grams is fatal.

These authors also note that henzoylated gelatin has a much more powerful reactivity than the homologous proteins in their relative precipitating power. Hopkins and Wormall also observed this effect in their phenylureidogelatin compounds. They found that in the precipitin and complement fixation reactions with phenyluredogelatin and the homologous serum protein compounds, that the zone of maximal precipitation or complete

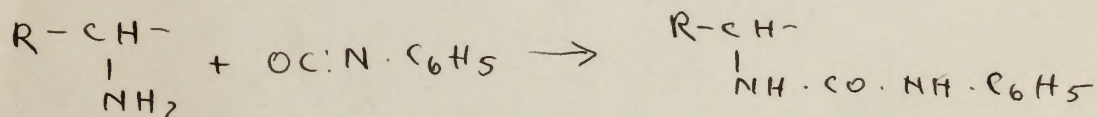
compounds, but also that the reaction, "do not show that non
antigenicity of gelatin is due solely to its solubility in
aromatic amino-acids." Other investigators have attributed the
theoretic aromatic groups were not able to react with gelatin on
gelatin but obtained only nonantigenic effects similar to heparin.
Lundquist found that distilled material was coupled
with gelatin gave crystalline with anti-horse-serum antibody
with a 1:1000 dilution. This gelatin compound was
initially inhibited reaction between the glass protein and the
antiserum using gelatin di-2-amino-2,5-dimethyl-6-sulfonic acid. He obtained the
same results with the homologous antibodies serum to horse.
Hershey and Corbridge found that benzoylated gelatin did
not give reactions with its antibodies, but give very strong re-
actions with antibodies for benzoylated horse serum and donkey-
labeled typhoid bacilli. The antibodies for benzoylated bacilli
reacted with a solution of gelatin containing 1 per cent of the
benzoyl reacted in a ratio of 1:10,000,000.
Benzoylated gelatin was strongly analogous to gelatin
after neutralized with the benzoylated typhoid bacilli, so that
a dose of 0.000015 grams is fatal.
These authors also note that benzoylated gelatin does not
show any essential reactivity than the homologous protein in
their relative crystallizing power. However, in Corbridge's
series this activity in their phosphorylation in horse serum, they
found that in the crystalline and found that the reaction
with benzoylated gelatin and the homologous serum protein was
greater than the rate of reaction with other proteins.

complement fixation is present in much more dilute solutions of the gelatin compounds. They note that the difference in activity is not dependent on the number of phenylureido groups coupled with the protein. No explanation is given for this phenomenon.

7a

Hopkins and Wormald introduced an entirely new method for coupling additional groups to the protein molecule. The older methods altered the structure of the protein by acids, alkalies, enzymes, nitration, iodization, etc. Landsteiner was the first to use azoproteins, produced by the reaction of various diazonium salts with proteins in alkaline solution. This is the method usually employed for introducing groups into the protein molecule. Coupling is supposed to take place only with the histidin and tyrosin in the protein.

The phenylisocyanate proteins can be prepared by coupling isocyanates to the proteins in a medium of pH 8. The reaction involves the free amino groups. Analysis of these proteins shows that the increase in the isocyanate content is accompanied by a corresponding decrease in the free amino nitrogen as found by bromin determination on p-bromophenylureido-proteins. The reaction may be represented as follows:



This method of introducing additional groups into the protein molecule has several advantages over the other methods used. The linkage closely resembles the peptid linkage, the

excess of isocyanate is rapidly destroyed, and its products are easily removed from the protein solution.

These authors obtained only combining effects with their phenylureidogelatin compounds. They got marked precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin. They also noted that precipitin reactions with the gelatin compounds are best carried out at 15-20°C or lower temperatures, since there is a tendency for the precipitates to dissolve at 37°. Despite injection^{ion} over a long period, they were unable to obtain procipitin or fixation reactions with phenylureido- and p-bromophenylureido gelatin using the homologous or heterologous phenylureido-proteins.

They conclude therefore that these gelatin compounds are non-antigenic and that the non-antigenicity of gelatin is not due solely to a deficiency in aromatic groups.

C. Discussion.

There is a peculiar lack of uniformity in the antigenic reactions of the synthethic gelatin compounds as reported by different investigators. In the first place Adant's¹ results are very hard to explain. Using an antidiazo-anilin-gelatin he observed precipitation with both gelatin and the homologous antigen although his gelatin was non-antigenic. This evidences that two antibodies were produced by gelatin-diazoanilin. It indicates that once having made gelatin antigenic by additional groups, gelatin itself will act as a precipitable hapten.

Hooker and Boyd⁶ were unable to get either precipitin or inhibition reactions with antidiazoarsanilic acid gelatin using gelatin itself.

A further complication is introduced when we consider the work of Bruynoghe and Vassiliadis.⁴ They obtain fixation with gelatin and not with the homologous antigen using antianilin gelatin. According to our present knowledge we would at least expect fixation with the homologous antigen. In view of these controversial findings the antigenicity of gelatin-diazo-anilin seems to be very doubtful. The author has therefore thought that a repetition of this work would yield interesting information.

According to the findings of Landsteiner;¹⁰ Medveczky and Uhrovits¹⁵ and Hopkins and Wormall,^{7b} there is no doubt that substituted gelatins are effective precipitable haptens. Hopkins^{7b} and Wormall reach the conclusion that to make gelatin antigenic

C. Discussion

There is a general lack of information in the literature regarding the specific effects of various compounds as reported by different investigators. In the first place, the results are very hard to compare. Using an anti-inflammatory agent as observed previously with both gelatin and the homologous antigen of which the gelatin was non-inflammatory. This indicates that the antibodies were produced by gelatin-immunization. It indicates that only having more gelatin in solution by additional groups, gelatin itself still act as a protein antigen.

Booker and Boyd are unable to get similar results in their reaction with anti-inflammatories with gelatin being negative results.

A further investigation is required when we consider the work of Murphy on "Gelatin". They obtain similar results and of this the conclusion might be drawn that gelatin, according to our present knowledge, is a protein antigen. However, with the non-inflammatory antigen, in the case of these non-inflammable antigens, the antigenicity of the antigen is due to the very nature of the antigen. The antigen is a protein compound.

There is a reaction of the antigen with the antibody.

Referring to the antigen of inflammation, the antigen is a protein compound and the antigen is a protein compound. The antigen is a protein compound and the antigen is a protein compound. The antigen is a protein compound and the antigen is a protein compound.

in the sense of engendering specific antibodies, the introduction of aromatic groups is not sufficient.

The evidence for the antigenicity of gelatin diazoarsanilic acid is not entirely satisfactory. Although positive fixation and precipitin reactions were obtained, these did not occur with the homologous but only with heterologous antigens.

Collagen is one of the most important of the proteins of the animal kingdom. It is the chief constituent of the connective tissues of the body. The organic material of the bones, tendons, cartilage and skin is largely composed of collagen.

Research has made a study of the structure of collagen and shown that it is a complex molecule. It is composed of only glycine, alanine, proline, lysine, arginine, hydroxyproline and hydroxylysine. All other amino acids are absent. The molecule is a triple helix. It is a very stable molecule and has a high melting point. It is a very important component of the animal kingdom.

Through study of the hydrolysis products of collagen the following data were obtained:

glycine	76.0%	proline	10.0%
alanine	8.0%	lysine	7.0%
arginine	0.5%	hydroxyproline	5.5%
hydroxylysine	5.5%		

in the sense of engineering specific studies, the intra-

action of chromatin structure is not sufficient.

The evidence for the significance of related associations

is not entirely satisfactory. Although positive reactions

and precipitin reactions were obtained, there was a lack of correlation

and homogeneity not only with the homologous antigens.

E. Some aspects of the chemistry of gelatin and collagen.

In this section an attempt has been made to summarize briefly some of the recent literature relating to the structure and chemical properties of gelatin and collagen. One of the very voluminous literature on the subject, the author attempted to bring out only those points that might have immunological significance.

Collagen is one of the most important of the proteins in the several connective tissues of the body. The organic material of the bones, tendons, cartilage and skin is largely composed of collagen.

19
SsadiKoff has made a study of the structure of collagen and comes to the conclusion that the collagen micelle contains only glycine, alanine, proline, leucine, arginine, lysine, asparagine, and glutamic acid, all other amino acids that might be found on analysis are impurities. He bases his conclusions on the result of x ray analysis, enzymatic action and the hydrolytic products of gelatin as determined by Dakin.³²

Through study of the hydrolysis products of gelatin Dakin found the following amino acids:

glycine	25.5%	proline	14.1%
alanine	8.7%	leucine	7.1%
arginine	8.2%	asparagine	3.4%
lysine	5.9%	glutamic acid	5.8%

E. Some aspects of the chemistry of gelatin and collagen.

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Collagen is one of the most important of the proteins in the several connective tissues of the body. The organic material of the bones, tendons, cartilage and skin is largely composed of collagen.

19

Goodfellow has made a study of the structure of collagen

and comes to the conclusion that the collagen molecule contains only glycine, alanine, proline, leucine, arginine, lysine, asparagine, and glutamic acid, all other amino acids that might be found in analysis are insignificant. He bases his conclusions on the result of x-ray analysis, enzymatic action and the hydrolytic products of collagen as determined by Berlin.

Through study of the hydrolytic products of gelatin Berlin

found the following amino acids:

glycine	33.5%	proline	14.1%
alanine	8.7%	leucine	7.1%
arginine	8.3%	asparagine	3.4%
lysine	5.9%	glutamic acid	5.0%

serin 0.04%

valin 0%

phenylalanin 1.4%

cystin 0%

hystidine 0.9%

tyrosin 0.01%

tryptophane 0%

Ssadikoff believes that in collagen the last group of amino acids as absent since they are present in very small percentage.

Furthurmore x ray analysis seem to bear out this point.⁵

The molecular unit as found by this method for collagen is 685.

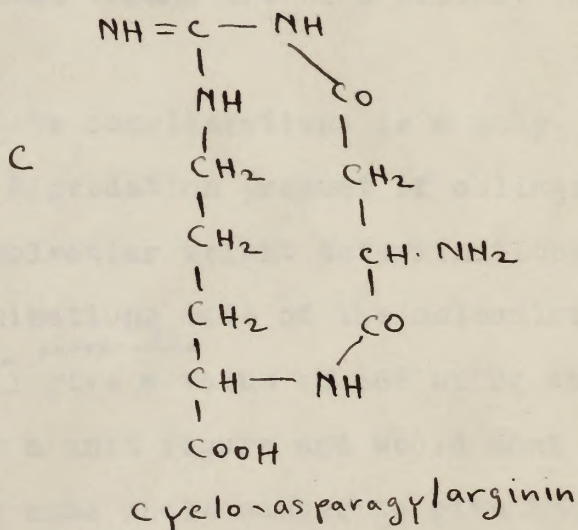
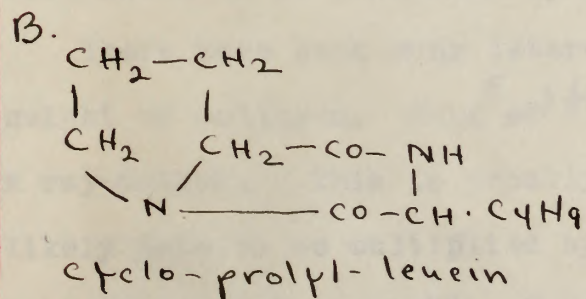
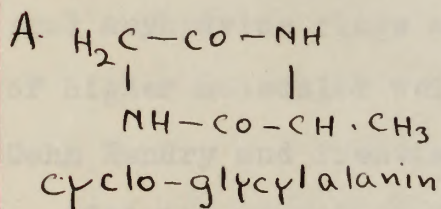
This indicates that the micelle of collagen is built up of four cyclopeptide linkages as follows:

Two molecules of cycloglyclalanin $114 + 114 = 228A$

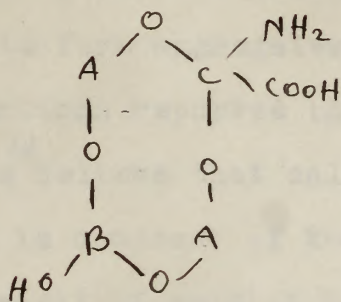
One molecule of cycloprolyl-leucin $= 198B$

One molecule of cyclo asparagyl-arginin $= 266C$

(or lysin) 692 Total



The formula for the collagen micelle would therefore be as follows:



Adjacent polypeptide rings are bound together by means of the enolic hydroxyl linkage. The free group (OH, NH, COOH) enable one unit to combine with another.

Collagen may therefore be considered as a cyclopeptide combined in an enolic anhydride ring. In the hydrolysis of collagen to gelatin a hydrolysis occurs with a breaking of the enol anhydride rings and the subsequent formation of aggregates of higher molecular weight. These views are also brought out by Cohn, Hendry, and Prentiss.⁵

Gelatin according to the above considerations is a polymerised rather than hydrolytic digredation product of collagen. This seems to be borne out by molecular weight determinations.

There have been many determinations made of the molecular weight of collagen. Cohn⁵ and his associates⁵ all give a value of 685 using the x ray method. This is probably a unit figure and would most likely have to be multiplied by some whole number to give the correct molecular weight. Herzog^{3a} found the molecular weight vary between 2000 and 2600 by determining the diffusion

coefficient of a polydispersed solution of collagen in m-cresol. This value may be accepted as a maximal ~~of~~ molecular weight since proteins tend to form aggregates in solution. There are many other determinations reported in the literature.

^{3d}
Herzog and Janke believe that collagen is not a single chemical entity but is composed of two substances.

⁹
The molecular weight of gelatin has been determined by Svedberg using the ultracentrifuge. He found that gelatin is a mixture of proteins varying in molecular weight from 11,000 to 70,000. He considers values below 11,000 as degradation products. His evidence seems to indicate that the true molecular weight of gelatin is 11,000 and that the higher values are merely molecular aggregations.

If we accept 2600 as the molecular weight of collagen and 11,000 as the molecular weight of gelatin, then in the change from collagen to gelatin a polymerization occurs instead of a hydrolytic breakdown as ordinarily assumed.

^{2o}
The action of enzymes on collagen furnish very important clues to its structure. Trypsin free from collagenase does not attack collagen. This is a characteristic of the action of trypsin on cyclic structures. It might also be noted at this point that ninhydrin, a reagent which reacts with proteins, polypeptids and alpha amino acids does not give a blue color with collagen. Pepsin however acts on collagen in acid medium to give non gelling peptised products. Collagenase free from trypsin peptises collagen in alkaline solution without forming

coefficient of a polydispersed solution of collagen in acetone. This value may be lower or as a maximum molecular weight since restricted to two aggregates in solution. There are many other determinations reported in the literature. Hargreaves and Jencks believe that collagen is not a single chemical entity but is composed of two substances. The molecular weight of collagen has been determined by Veberg using the ultracentrifuge. He found that collagen is a mixture of proteins varying in molecular weight from 11,000 to 17,000. He considers values below 11,000 as degradation products. His evidence seems to indicate that the true molecular weight of collagen is 11,000 and that the higher values are merely molecular aggregates. If we accept 11,000 as the molecular weight of collagen and 11,000 as the molecular weight of gelatin, then in the change from collagen to gelatin a polymerization occurs instead of a hydrolytic breakdown as ordinarily assumed. The action of enzymes on collagen furnish very important clues to its structure. Trypsin gives rise to peptides which are strong solvents. There is a characteristic of the action of trypsin on collagen. It might also be noted at this point that ribonuclease, a reagent which reacts with proteins, polypeptides and other amino acids does not give a blue color with collagen. Ribonuclease acts on collagen in solid solution. Five non gelatin peptides obtained. Gelatinases free from trypsin peptides collagen in alkaline solution without forming

amino acids.

3b

Collagens from different tissues do not have the same composition. It might be possible that collagen exhibits a tissue specificity. There seem to be chemical differences in the bone collagen of male and female animals.^{3c}

being added. 3p
 Cells from different classes do not have the same
 composition. It might be possible that cells exhibit a
 tissue specificity. There seem to be chemical differences in
 the composition of male and female animals.

F. Theoretical considerations

No satisfactory explanation has been advanced to date to explain the non-antigenicity of gelatin. In order to look at the problem intelligently we may well ask, to what factors in general do proteins owe their antigenicity? That knowledge we do have on the subject is very vague.

The first problem is the matter of definition. For as ^{23a} Wells notes in his review "An antigen is any substance which introduced into the tissues or circulating fluids of an animal causes the appearance in these fluids sooner or later of substances which specifically ^{react} ^{the} with ^{the} antigenic substance i.e. specific antibodies."

It is quite possible that antibodies may be produced within the cells and remain fixed and therefore not appear in the blood.

In order to exhibit antigenicity, the substance injected must be foreign to the tissues of the body so that appropriate defence mechanisms can be set up resulting in the formation of antibodies. As far as we know, gelatin is certainly foreign to the animal body. Collagen is not foreign to the animal body. However collagens from different animals have been shown to differ in chemical composition ^{3b} and might therefore be expected to stimulate the production of antibodies if injected into another animal.

Also antigenicity seems to be dependent on the size of the molecule. Gelatin contains varying proteins in molecular

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weight from about 11,000 to 70,000, which is of the order of magnitude of other antigenic proteins. Molecular aggregations do not seem necessary since in the concentrations used for immunizing, many proteins, egg albumin, for example, are mono-dispersed.

If the work on collagen can be confirmed, we may have to discard the idea that aromatic rings are necessary for antigenicity. It is possible that no particular amino acids are necessary for the antigenicity of proteins. There are claims in the literature that some complex carbohydrates are antigenic. In carbohydrates the building stones are usually simple monosaccharides. In any one carbohydrate the structural units do not have the diversity of the amino acids. Reasoning thus by analogy, it does not seem necessary to postulate the existence of certain amino acids in a protein in order to make it antigenic.

From purely chemical considerations it is difficult to explain the non-antigenicity of gelatin. During the change of collagen to gelatin no part of the molecule seems to be split away, the molecule is polymerised in fact. The question might then be asked, why should collagen be antigenic and gelatin not. Gelatin possibly lacks the proper internal arrangement of the amino acids. We know that under the influence of heat egg albumin is denatured and loses its antigenicity. Casein however does not lose its antigenic properties on heating. The "antigenic pattern" of some proteins is more stable than others. It might be possible to make gelatin antigenic by merely altering

weight from about 11,000 to 70,000, which is of the order of magnitude of other antigenic proteins. Molecular aggregation

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From purely chemical considerations it is difficult to explain the non-antigenicity of gelatin. During the change of gelatin to collagen a part of the molecule seems to be split away, the molecule is polymerized in fact. The question might then be asked, why should collagen be antigenic and gelatin not? Gelatin is easily broken the proper internal arrangement of the amino acids. We know that under the influence of heat egg albumin is denatured and loses its antigenicity. Gelatin however does not lose its antigenic properties on heating. The "antigenic pattern" of some proteins is more stable than others. It might be possible to make gelatin antigenic by merely altering

the configuration of the molecule by some physical means. It was thought that a plastein synthesis of a gelatin digest might give a possible antigenic rearrangement of the molecule.

²²Wastenys and Borsook however could not make a plastein from gelatin. It is also rather interesting that glue is a powerful atopen in human supersensitiveness.

It was thought that gelatin might be non antigenic due to racemization. Gelatin has a very high specific rotation ranging from -496.8 to -181.1 with wave lengths of light from 4339 to 6660 ^{3f} _{2f} Eggalbumin varies under some conditions from -83.9 to -27.5.

As a concluding thought, let us assume for the moment that by appropriate chemical procedures we could make gelatin fully antigenic. The results obtained would not necessarily be capable of general extension. Specificity and antigenicity are two concurrent factors. An antigenic substance is always specific. If specificity factors are absent then we cannot tell whether or not a substance is antigenic. We know that specificity varies with different proteins, and that it is possible to alter it by introducing additional groups. So that by solving the problem for gelatin we might ~~only~~ get results applicable only to that protein. To carry this reasoning further, the structural changes produced by the chemical reactions employed might make gelatin antigenic so that the introduction of additional groups might merely be an artifact. The additional group would then serve to change the specificity of an already antigenic molecule.

the denaturation of the molecule by some physical means. It was thought that a chemical synthesis of a gelatin digest might give a possible antigenic rearrangement of the molecule.

22
Stern and Borsack however could not make a gelatin from gelatin. It is also rather interesting that gelatin is a powerful adjuvant in human experiments.

It was thought that gelatin might be non antigenic due to its structure. Gelatin has a very high specific rotation ranging from -125 to -150, with wave lengths of light from 400 to 600 mμ. Gelatin varies under some conditions from -85 to -87.5.

As a concluding remark, let us assume for the moment that by appropriate chemical procedures we could make gelatin fully antigenic. The results obtained would not necessarily be capable of general extension. Specifically and antigenically we have two non-identical factors. An antigenic substance is always

specific. It specifically factors are absent & we cannot tell whether or not a substance is antigenic. We know that antigenicity varies with different proteins, and that it is possible to alter it by introducing additional groups. So that by solving the problem of gelatin we might get results applicable only

to that protein. To carry this reasoning further, the structural changes produced by the chemical reactions employed might make gelatin antigenic so that the introduction of additional groups might merely be an accident. The additional group would then serve to change the specificity of an already antigenic molecule.

II. Experimental work.

A. Preparation of nitro-proteins.

11

Landsteiner and Barron have demonstrated that if a racemised protein is nitrated its antigenic properties would be restored. Gelatin, from its method of preparation, may be considered a racemised protein. It was therefore thought that nitration might make gelatin antigenic.

13

After trying several modifications of Lieben's original procedure we prepared a nitro-gelatin in the following manner:

(1) Preparation of nitro-gelatin.

Twenty grams of gelatin (lot no 18613 Bacto Difco Brand controlled for non-antigenicity by Hooker and Boyd^b) was sifted into 100 cc. of concentrated nitric acid. Nitration was allowed to continue for ten minutes. The temperature during nitration varied from 64-70° C. The solution was immediately diluted to about 500 cc. with cold water. Solid ammonium sulphate was then added until precipitate was light yellow in color and resembled a sticky gum. It was suspended in 500 cc. of water and dissolved by adding alkali to a slight basic reaction of litmus. The resulting orange-colored solution was dialysed over night, subsequently sterilized by Berkefeld filtration and made isotonic with salt. The nitrogen content was found to be 3.68 mgs. of nitrogen per cc.

(2) Preparation of nitro-casein.

Twenty grams of casein (Pfandsteil, lot 6016) were sifted into 500 cc. of 20 per cent nitric acid and cooled under the tap during nitration. Small clumps were formed which did not

II. Preparation of Nitro-gelatin.

1. Preparation of Nitro-gelatin.

Handelman and Barton have demonstrated that if a reaction is initiated by the addition of a small amount of nitric acid, the reaction proceeds to completion. It was therefore thought that nitric acid might make gelatin nitratable.

13

After trying several modifications of Handelman's original procedure we prepared a nitro-gelatin in the following manner:

(1) Preparation of Nitro-gelatin.

Twenty grams of gelatin (for no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100) was dissolved in 100 cc. of concentrated nitric acid. The solution was allowed to stand for ten minutes. The temperature during this time varied from 65-70°C. The solution was immediately added to a beaker of water. The resulting mixture was stirred for ten minutes. It was then added to 500 cc. of water and stirred for ten minutes. It was then added to 1000 cc. of water and stirred for ten minutes. It was then added to 2000 cc. of water and stirred for ten minutes. It was then added to 4000 cc. of water and stirred for ten minutes. It was then added to 8000 cc. of water and stirred for ten minutes. It was then added to 16000 cc. of water and stirred for ten minutes. It was then added to 32000 cc. of water and stirred for ten minutes. It was then added to 64000 cc. of water and stirred for ten minutes. It was then added to 128000 cc. of water and stirred for ten minutes. It was then added to 256000 cc. of water and stirred for ten minutes. It was then added to 512000 cc. of water and stirred for ten minutes. It was then added to 1024000 cc. of water and stirred for ten minutes. It was then added to 2048000 cc. of water and stirred for ten minutes. It was then added to 4096000 cc. of water and stirred for ten minutes. It was then added to 8192000 cc. of water and stirred for ten minutes. It was then added to 16384000 cc. of water and stirred for ten minutes. It was then added to 32768000 cc. of water and stirred for ten minutes. It was then added to 65536000 cc. of water and stirred for ten minutes. It was then added to 131072000 cc. of water and stirred for ten minutes. It was then added to 262144000 cc. of water and stirred for ten minutes. It was then added to 524288000 cc. of water and stirred for ten minutes. It was then added to 1048576000 cc. of water and stirred for ten minutes. It was then added to 2097152000 cc. of water and stirred for ten minutes. It was then added to 4194304000 cc. of water and stirred for ten minutes. It was then added to 8388608000 cc. of water and stirred for ten minutes. It was then added to 16777216000 cc. of water and stirred for ten minutes. It was then added to 33554432000 cc. of water and stirred for ten minutes. It was then added to 67108864000 cc. of water and stirred for ten minutes. It was then added to 134217728000 cc. of water and stirred for ten minutes. It was then added to 268435456000 cc. of water and stirred for ten minutes. It was then added to 536870912000 cc. of water and stirred for ten minutes. It was then added to 1073741824000 cc. of water and stirred for ten minutes. It was then added to 2147483648000 cc. of water and stirred for ten minutes. It was then added to 4294967296000 cc. of water and stirred for ten minutes. It was then added to 8589934592000 cc. of water and stirred for ten minutes. It was then added to 17179869184000 cc. of water and stirred for ten minutes. It was then added to 34359738368000 cc. of water and stirred for ten minutes. It was then added to 68719476736000 cc. of water and stirred for ten minutes. It was then added to 137438953472000 cc. of water and stirred for ten minutes. It was then added to 274877906944000 cc. of water and stirred for ten minutes. It was then added to 549755813888000 cc. of water and stirred for ten minutes. It was then added to 1099511627776000 cc. of water and stirred for ten minutes. It was then added to 2199023255552000 cc. of water and stirred for ten minutes. It was then added to 4398046511104000 cc. of water and stirred for ten minutes. It was then added to 8796093022208000 cc. of water and stirred for ten minutes. It was then added to 17592186044416000 cc. of water and stirred for ten minutes. It was then added to 35184372088832000 cc. of water and stirred for ten minutes. It was then added to 70368744177664000 cc. of water and stirred for ten minutes. It was then added to 140737488355328000 cc. of water and stirred for ten minutes. It was then added to 281474976710656000 cc. of water and stirred for ten minutes. It was then added to 562949953421312000 cc. of water and stirred for ten minutes. It was then added to 1125899906842624000 cc. of water and stirred for ten minutes. It was then added to 2251799813685248000 cc. of water and stirred for ten minutes. It was then added to 4503599627370496000 cc. of water and stirred for ten minutes. It was then added to 9007199254740992000 cc. of water and stirred for ten minutes. It was then added to 18014398509481984000 cc. of water and stirred for ten minutes. It was then added to 36028797018963968000 cc. of water and stirred for ten minutes. It was then added to 72057594037927936000 cc. of water and stirred for ten minutes. It was then added to 144115188075855872000 cc. of water and stirred for ten minutes. It was then added to 288230376151711744000 cc. of water and stirred for ten minutes. It was then added to 576460752303423488000 cc. of water and stirred for ten minutes. It was then added to 1152921504606846976000 cc. of water and stirred for ten minutes. It was then added to 2305843009213693952000 cc. of water and stirred for ten minutes. It was then added to 4611686018427387904000 cc. of water and stirred for ten minutes. It was then added to 9223372036854775808000 cc. of water and stirred for ten minutes. It was then added to 18446744073709551616000 cc. of water and stirred for ten minutes. It was then added to 36893488147419103232000 cc. of water and stirred for ten minutes. It was then added to 73786976294838206464000 cc. of water and stirred for ten minutes. It was then added to 147573952589676412928000 cc. of water and stirred for ten minutes. It was then added to 295147905179352825856000 cc. of water and stirred for ten minutes. It was then added to 590295810358705651712000 cc. of water and stirred for ten minutes. It was then added to 1180591620717411303424000 cc. of water and stirred for ten minutes. It was then added to 2361183241434822606848000 cc. of water and stirred for ten minutes. It was then added to 4722366482869645213696000 cc. of water and stirred for ten minutes. It was then added to 9444732965739290427392000 cc. of water and stirred for ten minutes. It was then added to 18889465931478580854784000 cc. of water and stirred for ten minutes. It was then added to 37778931862957161709568000 cc. of water and stirred for ten minutes. It was then added to 75557863725914323419136000 cc. of water and stirred for ten minutes. It was then added to 151115727451828646838272000 cc. of water and stirred for ten minutes. It was then added to 302231454903657293676544000 cc. of water and stirred for ten minutes. It was then added to 604462909807314587353088000 cc. of water and stirred for ten minutes. It was then added to 1208925819614629174706176000 cc. of water and stirred for ten minutes. It was then added to 2417851639229258349412352000 cc. of water and stirred for ten minutes. It was then added to 4835703278458516698824704000 cc. of water and stirred for ten minutes. It was then added to 9671406556917033397649408000 cc. of water and stirred for ten minutes. It was then added to 19342813113834066795298816000 cc. of water and stirred for ten minutes. It was then added to 38685626227668133590597632000 cc. of water and stirred for ten minutes. It was then added to 77371252455336267181195264000 cc. of water and stirred for ten minutes. It was then added to 154742504910672534362390528000 cc. of water and stirred for ten minutes. It was then added to 309485009821345068724781056000 cc. of water and stirred for ten minutes. It was then added to 618970019642690137449562112000 cc. of water and stirred for ten minutes. It was then added to 1237940039285380274899124224000 cc. of water and stirred for ten minutes. It was then added to 2475880078570760549798248448000 cc. of water and stirred for ten minutes. It was then added to 4951760157141521099596496896000 cc. of water and stirred for ten minutes. It was then added to 9903520314283042199192993792000 cc. of water and stirred for ten minutes. It was then added to 19807040628566084398385987584000 cc. of water and stirred for ten minutes. It was then added to 39614081257132168796771975168000 cc. of water and stirred for ten minutes. It was then added to 79228162514264337593543950336000 cc. of water and stirred for ten minutes. It was then added to 158456325028528675187087900672000 cc. of water and stirred for ten minutes. It was then added to 316912650057057350374175801344000 cc. of water and stirred for ten minutes. It was then added to 633825300114114700748351602688000 cc. of water and stirred for ten minutes. It was then added to 1267650600228229401496703205376000 cc. of water and stirred for ten minutes. It was then added to 2535301200456458802993406410752000 cc. of water and stirred for ten minutes. It was then added to 5070602400912917605986812821504000 cc. of water and stirred for ten minutes. It was then added to 10141204801825835211973625643008000 cc. of water and stirred for ten minutes. It was then added to 20282409603651670423947251286016000 cc. of water and stirred for ten minutes. It was then added to 40564819207303340847894502572032000 cc. of water and stirred for ten minutes. It was then added to 81129638414606681695789005144064000 cc. of water and stirred for ten minutes. It was then added to 162259276829213363391578010288128000 cc. of water and stirred for ten minutes. It was then added to 324518553658426726783156020576256000 cc. of water and stirred for ten minutes. It was then added to 649037107316853453566312041152512000 cc. of water and stirred for ten minutes. It was then added to 1298074214633706907132624082305024000 cc. of water and stirred for ten minutes. It was then added to 2596148429267413814265248164610048000 cc. of water and stirred for ten minutes. It was then added to 5192296858534827628530496329220096000 cc. of water and stirred for ten minutes. It was then added to 10384593717069655257060992658440192000 cc. of water and stirred for ten minutes. It was then added to 20769187434139310514121985316880384000 cc. of water and stirred for ten minutes. It was then added to 41538374868278621028243970633760768000 cc. of water and stirred for ten minutes. It was then added to 83076749736557242056487941267521536000 cc. of water and stirred for ten minutes. It was then added to 166153499473114484112975882535043072000 cc. of water and stirred for ten minutes. It was then added to 332306998946228968225951765070086144000 cc. of water and stirred for ten minutes. It was then added to 664613997892457936451903530140172288000 cc. of water and stirred for ten minutes. It was then added to 1329227995784915872903807060280344576000 cc. of water and stirred for ten minutes. It was then added to 2658455991569831745807614120560689152000 cc. of water and stirred for ten minutes. It was then added to 5316911983139663491615228241121378304000 cc. of water and stirred for ten minutes. It was then added to 10633823966279326983230456482242756608000 cc. of water and stirred for ten minutes. It was then added to 21267647932558653966460912964485513216000 cc. of water and stirred for ten minutes. It was then added to 42535295865117307932921825928971026432000 cc. of water and stirred for ten minutes. It was then added to 85070591730234615865843651857942052864000 cc. of water and stirred for ten minutes. It was then added to 170141183460469231731687303715884105728000 cc. of water and stirred for ten minutes. It was then added to 340282366920938463463374607431768211456000 cc. of water and stirred for ten minutes. It was then added to 680564733841876926926749214863536422912000 cc. of water and stirred for ten minutes. It was then added to 1361129467683753853853498429727072845824000 cc. of water and stirred for ten minutes. It was then added to 2722258935367507707706996859454145691648000 cc. of water and stirred for ten minutes. It was then added to 5444517870735015415413993718908291383296000 cc. of water and stirred for ten minutes. It was then added to 10889035741470030830827987437816582766592000 cc. of water and stirred for ten minutes. It was then added to 21778071482940061661655974875633165533184000 cc. of water and stirred for ten minutes. It was then added to 43556142965880123323311949751266331066368000 cc. of water and stirred for ten minutes. It was then added to 87112285931760246646623899502532662132736000 cc. of water and stirred for ten minutes. It was then added to 174224571863520493293247799005065324265472000 cc. of water and stirred for ten minutes. It was then added to 348449143727040986586495598010130648530944000 cc. of water and stirred for ten minutes. It was then added to 696898287454081973172991196020261297061888000 cc. of water and stirred for ten minutes. It was then added to 1393796574908163946345982392040522594123776000 cc. of water and stirred for ten minutes. It was then added to 2787593149816327892691964784081045188247552000 cc. of water and stirred for ten minutes. It was then added to 5575186299632655785383929568162090376495104000 cc. of water and stirred for ten minutes. It was then added to 11150372599265311570767859136324180752990208000 cc. of water and stirred for ten minutes. It was then added to 22300745198530623141535718272648361505980416000 cc. of water and stirred for ten minutes. It was then added to 44601490397061246283071436545296723011960832000 cc. of water and stirred for ten minutes. It was then added to 89202980794122492566142873090593446023921664000 cc. of water and stirred for ten minutes. It was then added to 178405961588244985132285746181186892047843328000 cc. of water and stirred for ten minutes. It was then added to 356811923176489970264571492362373784095686656000 cc. of water and stirred for ten minutes. It was then added to 713623846352979940529142984724747568191373312000 cc. of water and stirred for ten minutes. It was then added to 1427247692705959881058285969449495136382746624000 cc. of water and stirred for ten minutes. It was then added to 2854495385411919762116571938898990272765493248000 cc. of water and stirred for ten minutes. It was then added to 5708990770823839524233143877797980545530986496000 cc. of water and stirred for ten minutes. It was then added to 11417981541647679048466287755595961091061972992000 cc. of water and stirred for ten minutes. It was then added to 22835963083295358096932575511191922182123945984000 cc. of water and stirred for ten minutes. It was then added to 45671926166590716193865151022383844364247891968000 cc. of water and stirred for ten minutes. It was then added to 91343852333181432387730302044767688728495783936000 cc. of water and stirred for ten minutes. It was then added to 182687704666362864775460604089535377456991567872000 cc. of water and stirred for ten minutes. It was then added to 365375409332725729550921208179070754913983135744000 cc. of water and stirred for ten minutes. It was then added to 730750818665451459101842416358141509827966271488000 cc. of water and stirred for ten minutes. It was then added to 1461501637330902918203684832716283019655932542976000 cc. of water and stirred for ten minutes. It was then added to 2923003274661805836407369665432566039311865085952000 cc. of water and stirred for ten minutes. It was then added to 5846006549323611672814739330865132078623730171904000 cc. of water and stirred for ten minutes. It was then added to 11692013098647223345629478661730264157247460343808000 cc. of water and stirred for ten minutes. It was then added to 23384026197294446691258957323460528314494920687616000 cc. of water and stirred for ten minutes. It was then added to 46768052394588893382517914646921056628989841375232000 cc. of water and stirred for ten minutes. It was then added to 93536104789177786765035829293842113257979682750464000 cc. of water and stirred for ten minutes. It was then added to 187072209578355573530071658587684226515959365500928000 cc. of water and stirred for ten minutes. It was then added to 374144419156711147060143317175368453031918731001856000 cc. of water and stirred for ten minutes. It was then added to 748288838313422294120286634350736906063837462003712000 cc. of water and stirred for ten minutes. It was then added to 1496577676626844588240573268701473812127674924007424000 cc. of water and stirred for ten minutes. It was then added to 2993155353253689176481146537402947624255349848014848000 cc. of water and stirred for ten minutes. It was then added to 5986310706507378352962293074805895248510699696029696000 cc. of water and stirred for ten minutes. It was then added to 11972621413014756705924586149611790497021399392059392000 cc. of water and stirred for ten minutes. It was then added to 23945242826029513411849172299223580994042798784118784000 cc. of water and stirred for ten minutes. It was then added to 47890485652059026823698344598447161988085597568237568000 cc. of water and stirred for ten minutes. It was then added to 95780971304118053647396689196894323976171195136475136000 cc. of water and stirred for ten minutes. It was then added to 191561942608236107294793378393788647952342390272950272000 cc. of water and stirred for ten minutes. It was then added to 383123885216472214589586756787577295904684780545900544000 cc. of water and stirred for ten minutes. It was then added to 766247770432944429179173513575154591809369561091801088000 cc. of water and stirred for ten minutes. It was then added to 1532495540865888858358347027150309183618739122183602176000 cc. of water and stirred for ten minutes. It was then added to 3064991081731777716716694054300618367237478243367204352000 cc. of water and stirred for ten minutes. It was then added to 6129982163463555433433388108601236734474956486734408704000 cc. of water and stirred for ten minutes. It was then added to 12259964326927110866866776217202473468949912973468817408000 cc. of water and stirred for ten minutes. It was then added to 24519928653854221733733552434404946937899825946937634816000 cc. of water and stirred for ten minutes. It was then added to 49039857307708443467467104868809893875799651893875269632000 cc. of water and stirred for ten minutes. It was then added to 98079714615416886934934209737619787751599303787750539264000 cc. of water and stirred for ten minutes. It was then added to 196159429230833773869868419475239575503198607575501078528000 cc. of water and stirred for ten minutes. It was then added to 392318858461667547739736838950479151006397215151002157056000 cc. of water and stirred for ten minutes. It was then added to 784637716923335095479473677900958302012794430302004314112000 cc. of water and stirred for ten minutes. It was then added to 1569275433846670190958947355801916604025588860604008628224000 cc. of water and stirred for ten minutes. It was then added to 3138550867693340381917894711603833208051177721208017256448000 cc. of water and stirred for ten minutes. It was then added to 6277101735386680763835789423207666416102355442416034512896000 cc. of water and stirred for ten minutes. It was then added to 12554203470773361527671578846415332832204710884832069025792000 cc. of water and stirred for ten minutes. It was then added to 25108406941546723055343157692830665664409421769664138051584000 cc. of water and stirred for ten minutes. It was then added to 50216813883093446110686315385661331328818843539328276103168000 cc. of water and stirred for ten minutes. It was then added to 100433627766186892221372630771322662657637687078656552206336000 cc. of water and stirred for ten minutes. It was then added to 200867255532373784442745261542645325315275374157313104412672000 cc. of water and stirred for ten minutes. It was then added to 401734511064747568885490523085290650630550748314626208825344000 cc. of water and stirred for ten minutes. It was then added to 803469022129495137770981046170581301261101496629252417650688000 cc. of water and stirred for ten minutes. It was then added to 1606938044258990275541962092341162602522202993258504835301376000 cc. of water and stirred for ten minutes. It was then added to 3213876088517980551083924184682325205044405986517009670602752000 cc. of water and stirred for ten minutes. It was then added to 6427752177035961102167848369364650410088811973034019341205504000 cc. of water and stirred for ten minutes. It was then added to 12855504354071922204335696738729300820177623946068038682411008000 cc. of water and stirred for ten minutes. It was then added to 25711008708143844408671393477458601640355247892136077364822016000 cc. of water and stirred for ten minutes. It was then added to 51422017416287688817342786954917203280710495784272154729644032000 cc. of water and stirred for ten minutes. It was then added to 102844034832575377634685573909834406561420991568544309459288064000 cc. of water and stirred for ten minutes. It was then added to 205688069665150755269371147819668813122841983137088618918576128000 cc. of water and stirred for ten minutes. It was then added to 411376139330301510538742295639337626245683966274177237837152256000 cc. of water and stirred for ten minutes. It was then added to 822752278660603021077484591278675252491367932548354475674304512000 cc. of water and stirred for ten minutes. It was then added to 1645504557321206042154969182557350504982735865096708951348609024000 cc. of water and stirred for ten minutes. It was then added to 3291009114642412084309938365114701009965471730193417902697218048000 cc. of water and stirred for ten minutes. It was then added to 6582018229284824168619876730229402019930943460386835805394436096000 cc. of water and stirred for ten minutes. It was then added to 13164036458569648337239753460458804039861886920773671610788872192000 cc. of water and stirred for ten minutes. It was then added to 26328072917139296674479506920917608079723773841547343221577744384000 cc. of water and stirred for ten minutes. It was then added to 52656145834278593348959013841835216159447547683094686443155488768000 cc. of water and stirred for ten minutes. It was then added to 105312291668557186697918027683670432318895095366189372886310977536000 cc. of water and stirred for ten minutes. It was then added to 210624583337114373395836055367340864637790190732378745772621955072000 cc. of water and stirred for ten minutes. It was then added to 421249166674228746791672110734681729275580381464757491545243910144000 cc. of water and stirred for ten minutes. It was then added to 84249833334845749358334422146936345855116076292951498309048782028

dissolve. The solution was colored a deep yellow. The clumps dissolved however, on the addition of alkali and the solution turned orange in color. The protein was precipitated by the addition of a minimal quantity of acid and then suspended in 250 cc. of water and dissolved by the addition of a minimal quantity of alkali. Since these solutions were only to be used as test antigens they were not sterilized.

(3) The preparation of nitro-albumin.

Twenty grams of albumin were sifted into 500 cc. of 1:2 nitric acid and cooled under the tap during nitration. The nitration formed an insoluble golden yellow precipitate of nitro-albumin which was filtered by suction and washed with 200 cc. of water. The washed precipitate was then suspended in the water and dissolved by the addition of alkali to a slight basic reaction to litmus. The solution turned orange red in color. The protein was reprecipitated with hydrochloric acid and again dissolved in the minimal quantity of alkali. The nitrogen content was 3.08 mgs. per cc.

B. Preparation of diazoparamino-benzoic acid proteins.

(1) The gelatin compounds.

Ten grams of gelatin were dissolved in 100 cc. of hot water. The solution was gelled in the ice box, diced, and mixed with 200 cc. of cold N/1 sodium carbonate.

Paraaminobenzoic acid, 1.37 gms. (5 times the theoretical 0.002.) was diazotized with N/2 sodium nitrate about (20 cc.) at 0-3 C, starch iodide paper was used as indicator. The cooled diazo compound was then added to the protein-carbonate mixture and allowed to couple over night in the ice box.

The cubes were then filtered off on cheese cloth, with cold distilled water, melted and the protein compound precipitated by the addition of 1 liter of acidified alcohol. The precipitate was then filtered and redissolved as before. The process was repeated twice. The protein solution was then filtered, subsequently sterilized by Berkefeld filtration and then made isotonic with sodium chloride.

(2) The preparation of diazoparaminobenzoic acid horse serum.

This preparation was made according to the directions of Landsteiner and Van der Scheer: ¹² Paraaminobenzoic acid, 1.77 gms, was dissolved in 10 cc of water and 5 cc of 7N HCl and diazotised as usual. The solution was diluted with ice water to a volume of 100 cc. and poured into 100 cc of horse serum to which 50cc. of normal sodium hydroxide had been added. Coupling was allowed to take place for ten minutes at about

Preparation of diacetylmorphine-morphine salt crystals.

(1) The following procedure:

Ten grams of diacetylmorphine were dissolved in 100 ml. of hot

water. The solution was cooled in the ice box, stirred, and

mixed with 200 cc. of soft 10% sodium carbonate.

Crystallization took place at 15°C. The crystalline

0.003% was dissolved with 1/2 sodium chloride about 20 ml.

of 0-3% sodium chloride water was added as indicated.

Cooled also sodium was then added to the protein-carbonate

mixture and allowed to settle over night in the ice box.

The solids were then filtered off on a glass filter, with

cold distilled water, washed and the filtrate removed completely

by the addition of 1 liter of distilled alcohol. The

precipitate was then filtered and retained in a beaker. The

precipitate was rapidly dried. The protein solution was then

filtered, evaporated to dryness by careful filtration and

then more isolated with added alcohol.

(2) The preparation of diacetylmorphine-morphine salt crystals.

This preparation was made according to the directions of

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Langbein and Van der Kooij. Crystallization took place at 15°C.

and dissolved in 10 cc of water and 10 ml of 0.3% sodium

chloride solution. The solution was filtered with 10 ml of water to a

volume of 100 ml. and passed into 100 cc of 1% sodium

which was at normal saline temperature and been added.

Crystallization was allowed to take place for two hours at about

0° C. The proteins were then precipitated with hydrochloric acid centrifuged down and suspended in a small quantity of water. On addition of normal sodium hydroxid and stirring the solution becomes very viscous and jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the protein.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol, the proteins were again brought into solution with alkali and, after dilution, reprecipitated with hydrochloric acid and centrifuged. The precipitate was then dissolved in water at slight alkaline reaction, made up to a volume of 190 cc., and sterilized by Berkfeld filtration and made isotonic with sodium chloride.

The proteins were then precipitated with hyposulfite and centrifuged from the suspension in a small quantity of water. In addition to normal sodium hyposulfite and stirring the formation becomes very viscous and jelly-like. A large quantity of alcohol was added and subsequently enough hyposulfite was added to dissolve the proteins.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol, the proteins were again brought into solution with alkali and, after filtration, reprecipitated with hyposulfite and centrifuged. The precipitate was then dissolved in water at slight alkaline reaction, made up to a volume of 100 cc., and centrifuged. Finally filtration and were treated with sodium chloride.

(C) Preparation of anilin-proteins.

(1) The gelatin compound.

In this preparation a simpler procedure was used. The writer is indebted to Dr. Matthew A. Derow for this procedure. Anilin, 0.93 grams, dissolved in 10 cc. of water with 2.5 cc of concentrated hydrochloric acid, was diazotized as previously described and coupled with gelatin. Instead of repeated precipitation the cubes are dialysed directly in a cheese cloth bag. The cubes act as their own membrane in dialysis and do not dissolve in cold tap water. After dialysis over night the cubes swelled to twice their original size. The cubes were then washed several times with distilled water and then melted, made up to a volume of 200 cc. and made isotonic with NaCl. Since gelatin solutions are very hard to filter through a Berkefeld, sterilization was accomplished by heating the gelatin for 45 minutes at 95°. The sterility of the protein was controlled by streaking agar slants and incubating at 20° and 37°.

(2) Preparation of anilin-horse-serum.

This preparation was made in the same way as the para-aminobenzoic acid compound except that 100 cc. of normal sodium carbonate was used instead alkali. Equivalent quantities were used throughout.

(C) Preparation of methyl-pyridine.

(i) The relative composition.

In this preparation a slightly more than 100 g. of the ester is required. Dr. Heston M. Jones for this preparation, methyl, 0.05 g. and, dissolved in 10 cc. of water with 0.5 cc. of concentrated hydrochloric acid, was dissolved as previously described and coupled with methyl. Instead of repeated precipitation the whole was dissolved directly in a phase of the. The tubes are left in their own membrane in 24 hours and 50 cc. of absolute alcohol is added for water. After dilution over night the tubes are filled of white water or clear. The tubes were then washed several times with distilled water and then dried, made up to a volume of 500 cc. and sealed. The tubes were then placed in a water bath and very hard to filter through a filter. The solution was accomplished by adding a certain amount of alcohol at 25°. The starting of the solution was controlled by breaking apart the tubes and the tubes at 25° and 27°.

(C) Preparation of methyl-pyridine.

This preparation was made in the same way as the previous one. The solution was made except that 100 cc. of water and 0.5 cc. of concentrated hydrochloric acid was used instead of 0.5 cc. of water. The tubes were sealed and the tubes were made 200 cc.

(D) Immunization of rabbits.

The following is a record of the injection schedule used for the nitrogelatin.

<u>Date</u>	<u>No. of rabbit</u>	<u>Volume injected</u>	<u>Route</u>
3/9/34	680,703,704	1.5 cc	Intravenous
3/13/34	" " "	2 cc	"
3/14/34	" " "	2 cc	"
3/20/34	680,704	3 cc	"
	703	3 cc	Intraabdominal
3/21/34	680,704	3 cc	"
	703	3 cc	"
3/22/34	680,704	3 cc	"
	703	3 cc	"
3/27/34	680,703,704	5 cc	"
4/1/34	680,703,704	5 cc	"
4/3/34	" " "	5 cc	"
4/10/34	Final bleeding		

The blood serum was tested for precipitins after 9 injections, using nitrogelatin, nitrocasein and nitroalbumin. Dilutions of the antigens were made from 10-100,000. No precipitins were demonstrated.

Three rabbits were injected with paraminobenzoic acid gelatin over a period of approximately four months with total of 30 doses. The following is a record of the injection schedule.

of 30 doses. The following is a record of the injection

relation over a period of approximately four months with total
Three rabbits were injected with parathyroid acid

skins were demonstrated.

skins of the rabbits were made from 10-100,000. No preser-
tion, using the parathyroid, citric acid and ribonucleic. Dif-
The blood serum was tested for parathyroid after 5-10 days

4/10/34 Pinal bleeding

Date	No. of rabbit	Volume injected	Route
3/9/34	380,703,704	1.5 cc	Intravenous
3/13/34	" "	3 cc	"
3/14/34	" "	3 cc	"
3/20/34	380,704	3 cc	"
3/21/34	703	3 cc	Intraperitoneal
3/21/34	380,704	3 cc	"
3/21/34	703	3 cc	"
3/22/34	380,704	3 cc	"
3/22/34	703	3 cc	"
3/27/34	380,703,704	5 cc	"
4/1/34	380,703,704	5 cc	"
4/3/34	" "	5 cc	"

for the experiment.

The following is a record of the injection schedule used

(D) Immunization of rabbits.

<u>Date</u>	<u>No. of rabbit</u>	<u>Volume injected</u>	<u>Route</u>
10/29/34	760,761,762	5 cc	Intravenous
10/30/34	" " "	"	"
11/8/34	" " "	"	"
11/9/34	" " "	"	"
11/10/34	" " "	"	"
11/16/34	" " "	1"cc	"
11/17/34	" " "	"	"
11/30/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/1/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/7/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/8/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/20/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/21/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/27/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
" " "	Trial bleeding		
12/28/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/3/34	760,761	1 cc	Intravenous

Date	No. of readings	Volume injected	Remarks
12/23/34	750.751	2 cc	Intravenous
12/23/34	" "	"	"
12/23/34	" "	"	"
12/23/34	" "	"	"
12/23/34	" "	"	"
12/23/34	" "	1" cc	"
12/23/34	" "	"	"
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
" " "	752	2 cc	Intrabdominal
12/23/34	750.751	1 cc	Intravenous
" " "	752	2 cc	Intrabdominal
" " "	752	2 cc	Intrabdominal

<u>Date</u>	<u>No. of rabbit</u>	<u>Volume injected</u>	<u>Route</u>
1/3/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/4/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/11/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/12/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/17/35	760,761,762	2 cc	Intraabdominal
1/18/35	" " "	" "	"
1/19/35	" " "	" "	"
1 week rest period			
2/1/35	760,761,762	1 cc	Intraabdominal
2/2/35	" " "	" "	"
2/7/35	" " "	$\frac{1}{2}$ cc	Intravenous
2/8/35	" " "	" "	"
2/9/35	" " "	" "	"
2/14/35	" " "	" "	"
2/15/35	" " "	" "	"
2/16/35	" " "	" "	"
2/22/35	Final bleeding		

After the fourteenth injection a trial bleeding was made and precipitin tests tried. Antigen dilution from 10 to 100,000 were made using gelatin paraminobenzoic acid and the horseserum compound. No intrafacial rings were observed after fifteen minutes or one hour at room temperature. The tubes were mixed

Date	No. 1 Rabbit	Volume injected	Route
1/3/35	750,751	1 cc	Intravenous
" " "	752	2 cc	Intracerebral
1/4/35	750,751	1 cc	Intravenous
" " "	752	2 cc	Intracerebral
1/11/35	750,751	1 cc	Intravenous
" " "	752	2 cc	Intracerebral
1/12/35	750,751	1 cc	Intravenous
" " "	752	2 cc	Intracerebral
1/17/35	750,751,752	2 cc	Intracerebral
1/18/35	" " "	" "	"
1/19/35	" " "	" "	"
I week rest period			
2/1/35	750,751,752	1 cc	Intracerebral
2/2/35	" " "	" "	"
2/7/35	" " "	1/2 cc	Intravenous
2/8/35	" " "	" "	"
2/9/35	" " "	" "	"
2/10/35	" " "	" "	"
2/11/35	" " "	" "	"
2/12/35	" " "	" "	"
2/13/35	" " "	" "	"
2/14/35	" " "	" "	"
2/15/35	" " "	" "	"
2/16/35	" " "	" "	"
2/17/35	" " "	" "	"

After the 17th injection a trial bleeding was made and protein levels noted. Another bleed from 15 to 100,000 were made using gelatin paraffinized acid and the following occurred. No intracerebral times were observed after fifteen minutes or one hour of room temperature. The blood was

and allowed to stand over night in the ice box. No precipitins could be found the next morning. The following morning precipitates were observed.

<u>Serum</u>		<u>Dilutions of horse serum paraaminobenzoic acid</u>				
		10	100	1000	10,000	100,000
761	Immune	+++	++	-	-	-
	Normal	++	++	-	-	-
762	Immune	+++	+++	±	-	-
	Normal	±	++	+	±	-

The plus signs indicate relative amounts of precipitate.

It was thought desirable to repeat these reactions. The tests were repeated using horse serum as an additional antigen. An attempt was made to duplicate the previous conditions in so far as possible. The results were completely negative. A subsequent precipitin test made after the final bleeding was also completely negative.

Three rabbits were also injected with analingelatin. The following is a record of the injection schedule.

<u>Date</u>	<u>Number of rabbit</u>	<u>Amount</u>	<u>Route</u>
12/20/34	763, 766, 767	$\frac{1}{2}$ cc	Intravenous
12/21/34	" " "	"	"
12/27/34	" " "	1 cc	"
12/28/34	" " "	"	"
1/3/35	" " "	"	"
1/4/35	" " "	"	"

and allowed to stand over night in the box. The precipitates would be found the next morning. The following morning precipitates were observed.

<u>Dilutions of horse serum antiserum to bacillus</u>					<u>Serum</u>	
100,000	10,000	1,000	100	10		
-	-	-	++	+++	Immune	V81
-	-	-	++	++	Normal	
-	-	±	+++	+++	Immune	V82
-	±	+	++	±	Normal	

The first rabbit relative to the results of the tests. It was thought desirable to repeat these reactions. The tests were repeated using for a serum an additional antigen. An attempt was made to duplicate the previous conditions in so far as possible. The results were completely negative. A subsequent precipitation test made after the final bleeding was also completely negative. Three rabbits were also infected with bacillus. The following is a record of the infection reactions.

<u>Date</u>	<u>Number of rabbit</u>	<u>Amount</u>	<u>Route</u>
12/20/34	723, 750, 757	1 cc	Intravenous
12/21/34	" " "	"	"
12/27/34	" " "	1 cc	"
12/28/34	" " "	"	"
1/3/35	" " "	"	"
1/4/35	" " "	"	"

<u>Date</u>	<u>Number of rabbit</u>	<u>Amount</u>	<u>Route</u>
1/11/35	763, 766, 767	1 cc	Intravenous
1/12/35	" " "	"	"
1/17/35	" " "	"	"
1/18/35	" " "	"	"
1/19/35	" " "	"	"
1/24/35	" " "	"	"
1/25/35	" " "	"	"
2/1/35	" " "	2 cc	Intraabdominal
2/2/35	" " "	"	"
2/7/35	" " "	"	"
2/8/35	" " "	"	"
2/9/35	" " "	"	"
2/14/35	" " "	"	"
2/15/35	" " "	"	"
2/16/35	" " "	"	"
2/23/35	Final bleeding		

Precipitin tests were tried using horse serum anilin and the
gelatin compound inductions of
 corresponding 10 to 100,000. The results were completely
 negative.

III. Summary.

Gelatin is non-antigenic. Some compounds have been prepared in which gelatin is claimed to be antigenic. Other investigators report only combining reactions with synthetic gelatin compounds. Aromatic amino acids are supposed to be necessary for antigenicity. Additional considerations about collagen introduce the possibility of a new concept. Analin, paraaminobenzoic acid and nitro gelatins have been prepared and injected. The results so far have been absolutely negative.

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IV. Bibliography.

1. Adant, M., "L'azoprotéine Gelatine-aniline"
Comp. rend. Soc. 1930 103 541
2. Arthus, Arch. Internat. de physiol. 1908 7 493
cited by Starin page 141
3. Biochemisches Handlexikon; XIV. Band (7. Ergänzungsband) 1933
Verlag von Julius Springer
 - a. page 253 Herzog R.O. Helevet. Chim. Acta II
1928 529
 - b. page 253 Knaggs J. Biochem. J. 1930 23 1308
 - c. page 253 Tadokoro T: J. Fac. Sci. Hokkaido
Imp. Univ. Seris III Chem. 1930 1 1
 - d. page 254 Herzog R.O. and Janke W.
Z. physik. Chem. B 12 228 1931
 - e. page 260 Dakin H.D. J. of Biol Chem 44 499 1920
 - f. page 265 Hewitt L.F. Biochem. J. 1927 21 216
4. Bruynoghe, R., and Vassiliadis, P.,
"Récherches sur les propriétés antigeniques
de la gélatine".
Compt. rend. Soc. Biol. 1930 103 541
5. Cohn, Hendry, Prentiss, J. Biol Chem. 1925 63 721
6. Hooker, S.B., and Boyd, W.C.,
"The antigenic Property of Gelatin-Diazo
Arsanilic Acid."
7. Hopkins, J. Jour. Immunol., 1933 24 141

IV. Bibliography.

1. Adams, M., "The properties of gelatin-sulfonates" Comp. Rend. Soc. Sci. 1933 103 541
2. Arthur, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
3. Blochmann, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
4. Bragg, R. A., and Vassiliadis, J., "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
5. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
6. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
7. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
8. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
9. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
10. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
11. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
12. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
13. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
14. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
15. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
16. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
17. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
18. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
19. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493
20. Carr, Henry, "The properties of gelatin-sulfonates" Internat. J. Physiol. 1938 7 493

7. Hopkins, J. H. and Wormald, A.,
 "Phenyl Isocyanate Protein Compounds and their
 Immunological properties."
 a. Biochem J., 1933 27 740
 b. ibid. 1706.
8. Kahn, R. L., and McNeil, A.,
 "Complement Fixation with Protein Substances."
 Jour. Immunol. 1918 3 277
9. Krishnamurto, K., and Swedberg, T.,
 "The Ultracentrifugal Study of Gelatin Solution."
 J. Am. Chem. Soc. 1930 52 2897
10. Landsteiner, K.
 "Ueber die Bedeutung der Proteinkomponente bei
 den Präzipitationsreaktionen der Azoproteine. XIII.
 Mitteilung über Antigene."
 Biochem. Ztschr. 1919 93 106
11. Landsteiner, K., and Barron, C.,
 "Ueber die Einwirkung von Säure und Lauge auf
 Serumweissantigenen (Restitution der Antigeneigen-
 schaft) IX. Mitteilung über Antigene."
 Ztschr. f. Immunitätsf. 1917 26 142
12. Landsteiner, K., and von der Scheer
 "The Influence of acid and Basic Groups on the
 Specificity of Azoproteins"
 J. Exp. Med. 1927 24 11045
13. Lieben, F.,
 "Über die Nitrierung einiger Eiweisskörper I"
 Biochem. Ztschr. 1924 145 535

14. Loiselleur, J., et Urbain, Ach.,
 "Sur les propriétés antigeniques du Collagène
 et leur modification sous l'action de l'eman-
 ation du Radium."
 Comp. rend. Soc. Biol. 1930 103 776
15. Medveczky, A., and Alhrovits, A.,
 "Immunitätsforschungen mit benzoylieten Antigenen."
 Ztschr. F. Immunitatsf. 1931 72 256
16. Nogeatte, J.,
 "Sur le callot artificiel de Collagène"
 Comp. rend. Soc. Biol. 1927 96 172
17. Pabes and Regazzoli.,
 R. acct. Fisiocril
221 page 557 cited by Starin, page 141
18. Ramsdell, S. G., and Walzer, M.
 "On Antigens; Antigenic Properties of Glue,
 Wheat and House Dust"
 Jour. Immunol. 1927 14 207
19. Ssadikoff, W.
 "Die Biochemischen Probleme bei der Lederher-
 stellung"
 Collegium 1926 356
20. Ssadikoff, W.
 "Ueber die Eigenschaften von Haut-und Sehnen-
 Kollagen und über ihr Verhalten gegenüber
 Fermenten"
 Collagen 1926 512

14. Hirschfeld, S., et Urbain, Ach.,
"Sur les propriétés antigéniques du collagène
et leur modification sous l'action de l'acide
acétique du collagène."
Comp. rend. Soc. Biol. 1930 103 776
15. Medvedev, A., and Alimov, A.,
"Immunitätsversuchen mit benzoylierten Antigenen."
Biochem. Z. Immunol. 1931 22 255
16. Koster, ...
"Sur le rôle antitoxique du collagène"
Comp. rend. Soc. Biol. 1929 92 172
17. Faber and Bergman,
A. Acad. Sci. Stockholm
SRI case 257 cited by Starin, page 141
18. Hammett, S. G., and Kaiser, A.,
"On antigenic properties of fibrin
ghost and whole cells"
Ann. Immunol. 1931 14 207
19. Sashkov, ...
"Die Blockaden des Proteins bei der Leber-
steifung"
Collection 1930 375
20. Sashkov, ...
"Über die Eigenschaften von Haut- und Sehnen-
leibern und über ihr Verhalten gegenüber
Toxinen"
Collection 1930 312

21. Starin, W. A., "The Antigenic Properties of Gelatin."
Jour. Infec. Dis., 1918 23 139
22. Wastenys, H. and Borsook, H.
"Enzymatic Synthesis of Proteins I"
J. of Biol. Chem 1924 62 23
23. Wells, H. G. "The Chemical Aspects of Immunity"
The Chemical Catalog Company
a. 26 b. 27 c. 32
24. Wells, H. G.
"The Nature of the Poisonous Element of Proteins
that is Concerned in the Reaction of Hypersensitization"
Jour. Amer. Med. Assoc. 1908 50 527

21. Sterlin, ... "The Latent Properties of Germanium."

Jour. Amer. Chem. Soc., 1918 22 139

22. Wenzel, H. and Horrocks, H.

"Magnetic Anisotropy of Crystals I"

J. Chem. Phys., 1934 2 23

23. Allen, H. G. "The Nature of the Polymers of Ethylene"

The Chemical Catalog Company

N. Y. N. Y. 27 2. 23

24. Allen, H. G.

"The Nature of the Polymers of Ethylene"

that is contained in the reaction of hydrogen

chloride"

Jour. Amer. Chem. Soc., 1908 30 237

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